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Antibiotic-Dependent Correlation Between Drug-Induced Killing and Loss of Luminescence in *Streptococcus gordonii* Expressing Luciferase

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ABSTRACT

Measuring antibiotic-induced killing relies on time-consuming biological tests. The firefly luciferase gene (*luc*) was successfully used as a reporter gene to assess antibiotic efficacy rapidly in slow-growing *Mycobacterium tuberculosis*. We tested whether *luc* expression could also provide a rapid evaluation of bactericidal drugs in *Streptococcus gordonii*. The suicide vectors pFW5luc and a modified version of pJDC9 carrying a promoterless *luc* gene were used to construct transcriptional-fusion mutants. One mutant susceptible to penicillin-induced killing (LMI2) and three penicillin-tolerant derivatives (LMI103, LMI104, and LMI105) producing luciferase under independent streptococcal promoters were tested. The correlation between antibiotic-induced killing and luminescence was determined with mechanistically unrelated drugs. Chloramphenicol (20 times the MIC) inhibited bacterial growth. In parallel, luciferase stopped increasing and remained stable, as determined by luminescence and Western blots. Ciprofloxacin (200 times the MIC) rapidly killed 1.5 $\log_{10}$ CFU/ml in 2–4 hr. Luminescence decreased simultaneously by 10-fold. In contrast, penicillin (200 times the MIC) gave discordant results. Although killing was slow ($\leq$0.5 $\log_{10}$ CFU/ml in 2 hr), luminescence dropped abruptly by 50–100-times in the same time. Inactivating penicillin with penicillinase restored luminescence, irrespective of viable counts. This was not due to altered luciferase expression or stability, suggesting some kind of post-translational modification. Luciferase shares homology with aminoacyl-tRNA synthetase and acyl-CoA ligase, which might be regulated by macromolecule synthesis and hence affected in penicillin-inhibited cells. Because of resemblance, luciferase might be down-regulated simultaneously. Luminescence cannot be universally used to predict antibiotic-induced killing. Thus, introducing reporter enzymes sharing mechanistic similarities with normal metabolic reactions might reveal other effects than those expected.

INTRODUCTION

Testing the activity of antibacterial drugs includes determining both their bacteriostatic and bactericidal effects. The bacteriostatic activity is quantified by their minimal inhibitory concentration (MIC).[1] The bactericidal activity assesses their ability to block irreversibly the resumption of cell division after removal of the drug from the medium. Both determinations are useful in predicting antibiotic treatment success. Being bactericidal is an important antibiotic property in circumstances of altered host defenses. This includes infections in the neutropenic patient and infections confined to so-called therapeutic sanctuaries, such as in infectious endocarditis and bacterial meningitis.

However, determination of these two parameters utilizes microbial growth as readout. Therefore, depending on the bacterial doubling time, it may take days to weeks to obtain the results. Attempting to circumvent this limitation Jacobs *et al.*[15] introduced the firefly luciferase gene *(luc)* into *Mycobacterium tuberculosis* and determined the luminescence of bacteria in the absence or presence of antibiotics. The *luc* product is a 62-kDa protein that does not require post-translational processing.[5,34] Thus, it can be functionally expressed by a variety of eukaryotic and prokaryotic cells.[11,15] It generates light by oxidizing beetle luciferin in an ATP-dependent manner, and has one of the highest quantum yields.[28] This makes it an excellent sensitive real-time reporter for assessing the energy level of the recipient bacterium.

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In their experiment, Jacobs *et al.* used a broad-spectrum lytic phage carrying the *luc* gene to infect *M. tuberculosis*.[15] Upon infection, recipient bacteria transiently transcribed the phage-encoded *luc* gene and emitted a burst of light. When antibiotics were added to the culture, the burst of light was inhibited in susceptible bacteria, but persisted in resistant organisms. Thus, the system allowed the discrimination between drug-susceptible and drug-resistant *M. tuberculosis* within hours as compared to days or weeks with conventional methods.

Recently, the system was improved by constructing a stable *luc*-positive lysogen in *M. smegmatis*,[27] to facilitate screening of new antimycobacterial drugs. Firefly luciferase was successfully used by Loimaranta *et al.*[17] and Tenhami *et al.*[30] to test the bacteriostatic effect of a variety of antibiotics against *Streptococcus mutans* and *Staphylococcus aureus*, respectively. However, none of the reported studies tested the activity of luciferase to predict antibiotic-induced killing.

Luciferase has a few theoretical features making it a suitable candidate to rapidly assess antibiotic-induced killing. First, intracellular luciferase activity requires the reporter organism to be physically intact. Thus, it would readily detect killing due to drug-induced bacterial lysis. Second, light emission requires the microorganism to produce ATP. Therefore, any membrane or metabolic alterations resulting in de-energizing the bacterial cell—a kind of alteration that is expected in dead cells—would be detected by a switch off of luminescence. Third, measuring luminescence in bacteria is technically easy and instantaneous. Thus, it would generate an online profile that could detect both lysis-independent and lysis-dependent killing in a much more rapid way than subculturing bacteria on agar plates as in standard time-kill experiments.[1]

The present experiments explored this possibility using an isogenic pair of luciferase-positive *Streptococcus gordonii* that were either susceptible or tolerant to penicillin-induced killing. Mechanistically unrelated drugs, including chloramphenicol, ciprofloxacin, and penicillin, were used. The *luc*-reporter system was tested under independent streptococcal promoters after insertion into the streptococcus.

MATERIALS AND METHODS

Microorganisms and growth conditions

Streptococcus gordonii Challis[25] and its various transformants are described in Table 1. *Escherichia coli* DH5α was used for plasmid propagation. Streptococci were grown at 37°C either in brain heart infusion broth (BHI; Difco laboratories, Detroit, MI) without aeration, or on Columbia agar (Becton Dickinson Microbiology Systems, Cockeysville, MD) supplemented with 3% blood. *E. coli* were grown in Luria Bertani (LB) broth or on LB agar. Growth of the cultures was followed both by optical density at a wavelength of 620 nm (OD_{620}) with a spectrophotometer (Sequoia-Turner, Montainville, CA) and

TABLE 1. BACTERIAL STRAINS USED IN THE STUDY AND SUSCEPTIBILITY TO PENICILLIN-INDUCED KILLING

S. gordonii *strains*	*Relevant genotype*	*Phenotype/utilization*	*Penicillin-induced killing (log CFU/ml)*[a]			*Source or reference*
			2 hr	*4 hr*	*24 hr*	
Challis (parent)	Wild type	Reference strain	0.5	1	3–4	[2,25]
LMI 2	Parent Ω pFWD5luc	Sp^r Luc^+/expresses *luc* under a chromosomal promoter	0.5	1	3–4	This work
Tol1[c]	Penicillin-tolerant mutant of *S. gordonii*	Tol^+ Sm^r/selected by repeated penicillin passages	0.2	0–0.5	0.5–1	[2]
Tol103[b]	Tol1 *arcB*::*luc-erm*	Tol^+ Luc^+ Em^r Sm^r/expresses *luc* under the *arc* promoter	0.2	0–0.5	0.5–1	[2]
Tol104[c]	Tol1 Ω pFWD5luc	Tol^+ Luc^+ Sp^r	0.2	0–0.5	0.5–1	This work
Tol105[d]	Tol1 *scaA*::*luc-erm*	Tol^+ Luc^+ Em^r/expresses *luc* under the *sca* promoter	0.2	0–0.5	0.5–1	[2,3]

Sp^r, Spectinomycin resistant; Em^r, erythromycin resistant; Sm^r, streptomycin resistant; *arc*, arginine deiminase operon; *scaA*, Mn-binding membrane lipoprotein gene.[3]

[a]Loss of viability (in log CFU/ml) after addition of 200× the MIC of penicillin (final concentration) to broth cultures of *S. gordonii*.

[b]Tol1 mutant containing a *luc* transcriptional-fusion reporter inserted into the *arcB* gene by means of the pJDC9 erm^+ suicide vector.[2]

[c]The pFWD5luc insert of mutant LMI2 was introduced into the Tol1 chromosome by transformation of Tol1 with LMI2 chromosomal DNA.

[d]Tol1 mutant containing a *luc* transcriptional-fusion reporter inserted into the *scaA* gene by means of the pJDC9 erm^+ suicide vector.[2,3]

by viable counts on agar plates. When appropriate, antibiotics were added to the medium at the following concentrations: spectinomycin (Sp) either 100 mg/L (for *E. coli*) or 500 mg/L (for *S. gordonii*), erythromycin (Em) 5 mg/L, penicillin (Pn) 0.8 mg/L, chloramphenicol (Cm) 640 mg/L, and ciprofloxacin (Cp) 200 mg/L. This corresponded to 200 times the MIC of penicillin and ciprofloxacin and 20 times the MIC of chloramphenicol for the test *S. gordonii*. Bacterial stocks were stored at −70°C in broth supplemented with 10% (vol/vol) glycerol.

Antibiotics and chemicals

Penicillin G was purchased from Aventis AG (Zurich, Switzerland); ciprofloxacin was purchased from Bayer AG (Wuppertal, Germany); D-luciferin, recombinant firefly luciferase and rabbit anti-luciferase antibodies were purchased from Promega (Promega Corporation, Madison, WI); DNA-modifying enzymes were purchased from Gibco Life Technologies (Gaithersburg, MD) and used according to the manufacturer's recommendations. All other chemicals were reagent-grade, commercially available products.

Antibiotic susceptibility and time-kill curves

The MICs of the test antibiotics were determined by a standard macrodilution method.[1] Time-kill curves were determined by adding appropriate concentrations of antibiotics to bacterial cultures in the exponential phase of growth at an OD_{620} of 0.2.[2] At various times before and after drug addition, samples were removed and spread on agar plates for colony count. Antibiotic carryover was avoided as described previously.[7,8] Colonies were counted after 48 hr of incubation at 37°C.

Construction of a luciferase transcriptional-fusion reporter system in S. gordonii

DNA and competent cells were prepared by already-described methods.[2,25,26] Plasmids were purified with a plasmid purification kit (Qiagen GmbH., Hilden, Germany). To insert the *Photinus pyralis* firefly luciferase (*luc*) gene into the *S. gordonii* chromosome, we used the suicide vector pFW5luc (kindly provided by A. Podbielski, University of Ulm, Ulm, Germany). This vector is derived from previous constructs (pFW-phoA and pFW-gfp) used in promoter-probe experiments.[22] It contains the pUC19 origin for replication in *E. coli* and a spectinomycin-resistance (Sp^r) marker. In addition, it carries multicloning site located upstream of a promoterless *luc* gene, containing its ribosome-binding site. This makes the construct suitable for transcriptional-fusion experiments.[12,23] pFW5luc cannot replicate autonomously in *S. gordonii,* but can express Sp^r provided that it is inserted into the chromosome. To achieve integration, a *Sau*3A digest of the recipient's chromosome was ligated at the *Bam*HI site of the vector, just upstream of the *luc* gene. The mixture of plasmid chimera was transformed into *S. gordonii*-competent cells. Integration of the construct occurred by insertion-duplication.[24] If the streptococcal DNA ligated upstream of *luc* contained a promoter sequence, then *luc* was expected to be expressed by the recombinant.[29] Transformants were selected on Sp-containing agar. Resistant colonies were picked, grown in antibiotic-containing liquid cultures, and screened for light emission. Additional constructs using a modified pJDC9 suicide vector were also used (Table 1).[2,3]

Determination of light emission in whole cells

Light emission was measured by slight modifications of a published method.[15] Tubes containing fresh prewarmed BHI broth were inoculated with 1/100 (vol/vol) from an overnight culture and growth was followed as described. At several times during logarithmic growth or after antibiotic addition, 100-μl samples of the cultures were removed, added to 5 ml of polystyrene (75 × 12-mm) tubes containing 250 μl of sodium citrate (pH 5.5) and equilibrated for 3 min at room temperature. A pH of 5.5 was chosen to inactivate luciferase that might leak from the cell into the extracellular milieu.[6] Immediately before light measurement, 50 μl of 1 mM beetle D-luciferin (Promega Corporation, Madison, WI) diluted in 25 mM glycylglycine and 15 mM $MgSO_4$ (GB buffer) were added to the mixture. The tubes were transferred into the luminometer (Luminat LB9501, EG & G Berchtold, Bad Wildbad, Germany) and the kinetics of light emission were followed over 5 min. During the exponential growth phase, light emitted by the bacteria peaked within the first 20 sec of luciferase addition, and drastically decreased thereafter to reach a plateau after 5 min. In the present experiments, peak luminescence was recorded and always determined within the first 20 sec of light emission. Luminescence was expressed in relative light unit per second (RLU/sec).

Determination of light emission in bacterial crude extracts

To evaluate the functionality of luciferase taken out of the intracellular milieu, light emission was also measured in crude extracts of bacteria. The microorganisms were grown in 500-ml flasks. Samples (50–100 ml) were removed at various times after drug addition and chilled at 4°C; the bacteria were recovered and washed twice in 0.9% NaCl by centrifugation. The pellets were resuspended in 1 ml of a 50 mM KPO_4 pH 7.4 and 10 mM of $MgSO_4$ solution. Specifically using 10 mM of $MgSO_4$ was important both because other anions (e.g., $MgCl_2$) inhibit luciferase activity, and higher salt concentrations (e.g., ≥100 mM) shift light emission from yellow-green to red.[6] The cells were mechanically broken (>90% as assessed by phase contrast microscopy) as described.[18] Large debris and nonlysed cells were removed by low-speed centrifugation (2000 rpm for 10 min). Protein concentration in the supernatant was determined by the bicinchoninic acid (BCA) protein assay (Pierce Chemical, Rockford, IL). Samples were stored at −30°C.

For light emission, 50 μl of a cell extract containing 5 μg of protein was added to 200 μl of GB buffer (as above) supplemented with 4 mM of EGTA (final concentration) and 9 μl of a 400 mM solution of ATP. After mixing, the tubes were equilibrated for 3 min at room temperature before being transferred into the luminometer. D-Luciferin (100 μl) was added. In contrast to the "flash light" kinetics observed in exponentially growing bacteria, light emission was stable in this *"ex vivo"* condition. Therefore, light output was measured starting 0.2 sec after D-luciferin addition and integrated over a 10-sec period of time. Recombinant luciferase was used as a control.

Luciferase detection by Western blot

To test for possible intracellular luciferase degradation, 10 μg of protein from crude extracts was separated by electrophoresis on an 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel.[16] The proteins were transferred overnight (at 4°C) onto an Immobilon-P PVDF membrane (Millipore Corporation, Bedford, MA) using a Mini-Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories, Hercules, CA) and a constant voltage of 35 V. The membranes were incubated with a final concentration of 0.5 mg/L of anti-luciferase polyclonal rabbit antibodies for 4 hr. After repeated rinsing with PBS and 0.2% Tween, the immunoblots were incubated for 1 hr with a 1:50,000 dilution of goat anti-rabbit antibodies coupled with alkaline phosphatase (Pierce). The bands were revealed by the BCIP (5-bromo-4-chloro-3-indol-1-phosphate-*p*-toluidine)-nitroblue tetrazolium method (AP development Reagent; Bio-Rad). Recombinant luciferase was used as a control.

RESULT

Construction of a luciferase transcriptional-fusion reporter system

Competent *S. gordonii* cells were treated with 1 μg/ml (final concentration) of pFWD5luc chimera containing random *Sau3*A chromosomal fragments. The transformation rate to Sp^r was 10^{-6}. Twelve resistant mutants were obtained in two experiments. Four of them (33%) expressed luciferase. The frequency of transcriptional fusion was comparable to previous studies using this random-insertion technique.[31]

One Sp^r mutant (LMI 2) expressing high luciferase activity over the whole exponential growth phase was further studied. Figure 1 depicts the correlation between viable counts and light emission during growth, and shows that luminescence correlated with viable counts. Similar observations were made with the luminescent tolerant derivatives Tol103, Tol104, and Tol105 used as controls (Table 1).[2,3]

The stability of the chromosomal *luc* insert was tested by serial passages in antibiotic-free broth followed by plating on selective agar. Seventy-five percent of LMI 2 had lost their Sp^r marker after ~35 generation times. Therefore, spectinomycin was kept in the medium in subsequent experiments. The presence of the drug did not alter bacterial growth or antibiotic-induced killing (data not shown). No attempt was made to determine the exact mechanism of this instability.

Finally, chain length variation could yield discordant results between luminescence and colony counts. Variation in chain length was scored by phase-contrast microscopy. *S. gordonii* formed short chains of 2 to 30 individuals that did not vary between the different isolates (Table 1) and was not affected by antibiotic treatment.

Titration of the system

Luciferase activity depends on several factors, including the intracellular amount of the protein, its stability after antibiotic addition, ATP concentration, and pH. Because these parameters may change independently of cell viability,[10,17] it was important to test the system in various physiological conditions

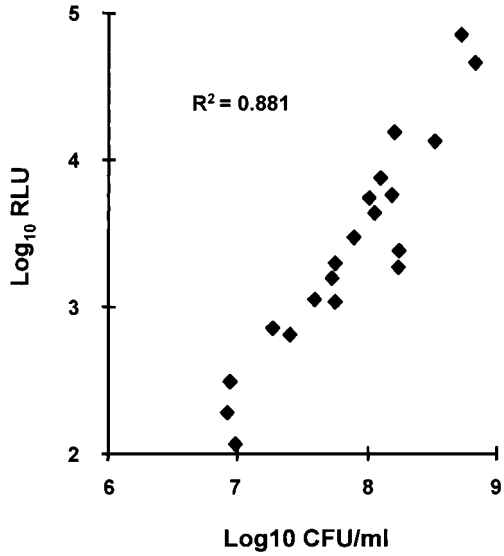


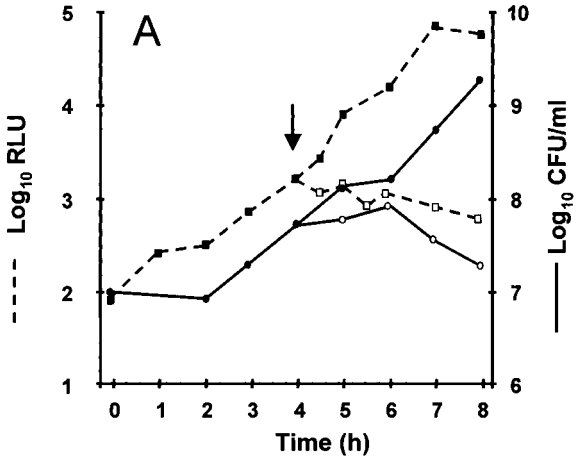
FIG. 1. Correlation between luminescence and bacterial growth in mutant LMI 2. Bacteria were grown in liquid cultures and sampled at various times for both viable counts and light emission. The data summarize a total of three independent experiments. Luminescence is expressed in relative light units (RLU) per second.

before running bactericidal experiments. First, we tested light emission during normal growth and after inhibition of protein synthesis with 20 times the MIC of chloramphenicol. Figure 2A indicates that luminescence was proportional to bacterial growth. After addition of chloramphenicol, bacterial growth came to a halt and viable counts marginally decreased over the following 4 hr. This was accompanied by a parallel leveling of light emission, which remained proportional to the viable counts. Thus, "protein-inhibited" but viable cells expressed luminescence for a prolonged period of time.

Second, we tested the physical and functional integrity of intracellular luciferase after chloramphenicol addition. Cell extracts were prepared and analyzed both by Western blot and by an *"ex vivo"* luminescence assay. In this condition, light emission depended on the exogenous adjustment of ATP and pH, thus bypassing possible intracellular alterations. Figure 2B indicates that luciferase remained physically and functionally unaltered for several hours after inhibition of protein synthesis. Thus, the stability and functionality of intracellular luciferase remained stable for several hours in nonkilled cells.

Luciferase activity in bioluminescent S. gordonii *treated with ciprofloxacin and penicillin*

The experiments were repeated with ciprofloxacin and penicillin, two bactericidal drugs with different modes of action (Fig. 3). After addition of 200 times the MIC of ciprofloxacin, the cultures' viable counts rapidly decreased by approximately 2 log CFU/ml over 4 hr (Fig. 3A). During the same time, the cultures' luminescence decreased in parallel by about 10 times, demonstrating some correlation between viable counts and light emission.



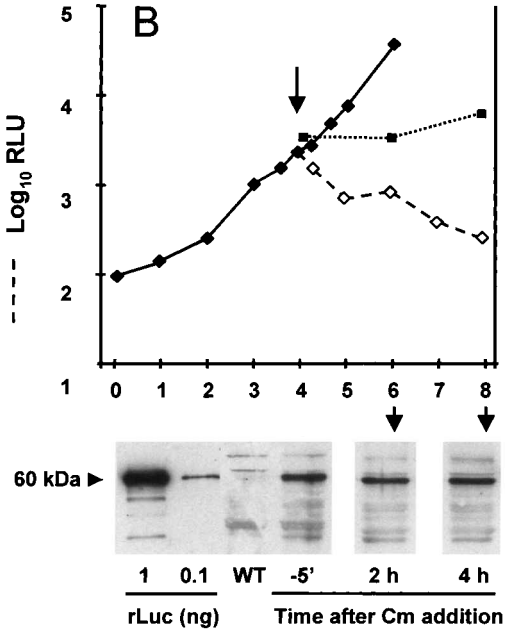


FIG. 2. Correlation between luminescence and viable counts in whole cultures (**A**) and cell extracts (**B**) before and after the addition of 20 times the MIC of chloramphenicol. The arrows indicate the time of drug addition. (**A**) Viable counts and luminescence of either untreated (closed symbols) or chloramphenicol-treated cultures (open symbols). The upper part of **B** depicts the luminescence of untreated (closed diamonds) or treated cultures (open symbols), as well as the luminescence of cell extracts as determined *ex vivo* (closed squares). The lower part of **B** presents the amount of luciferase detected by Western blot with either recombinant luciferase (rLuc), cell extracts of the wild-type parent (WT), or cell extracts of mutant LIM 2 (Table 1) prepared 5 min before, or 2 and 4 hr after drug addition. It can be seen that luciferase was quantitatively and functionally stable during chloramphenicol treatment.

After addition of 200 times the MIC of penicillin, the viable counts decreased at an average of 1 Log_{10} CFU/ml per 4–6 hr, reaching 3–4 orders of magnitude after 24 hr (Fig. 3B and Table 1). This was slightly slower (by about 1 Log_{10} at 4 hr) than in a previous report by Caldelari *et al.*[2] The difference came from the fact that Caldelari *et al.* added streptomycin to the growth media to prevent bacterial contamination during serial passages. Although their *S. gordonii* was streptomycin resistant, the addition of streptomycin slightly increased the rate of penicillin-induced killing.

In the present experiments, in spite of relatively slow killing, luminescence sharply dropped by ≥10 times within the first hour of penicillin treatment, reaching background levels within 2 hr (Fig. 3B). This discordance between slow viable loss and rapid switch off of luminescence was repeatedly observed in six independent experiments with mutant LMI 2.

To determine further whether the phenomenon was related to penicillin treatment rather than other coincidental phenomena, the experiments were repeated with three tolerant derivatives of *S. gordonii* carrying a *luc* reporter insert in indepen-

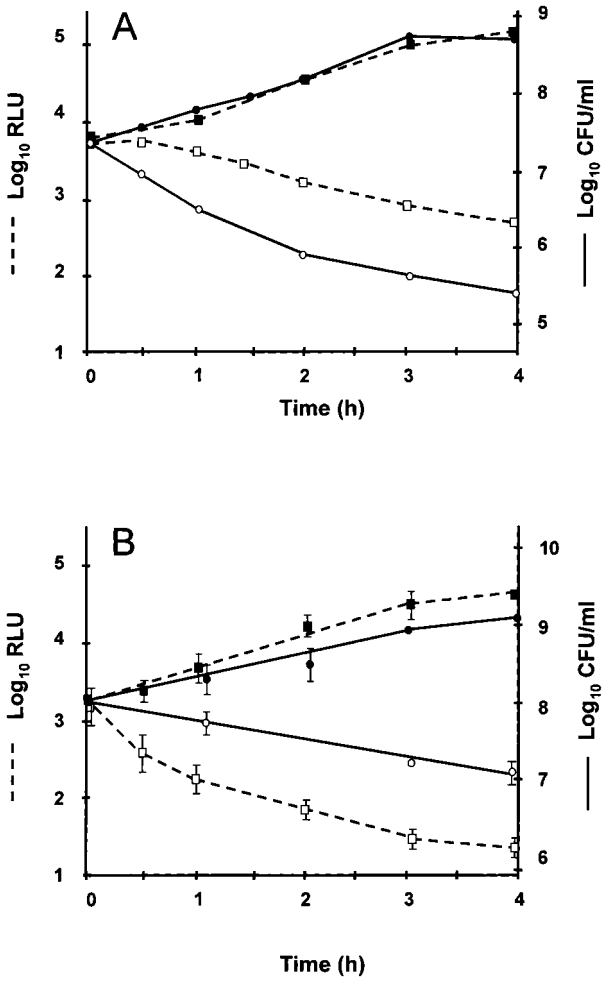


FIG. 3. Correlation between luminescence and bacterial growth in mutant LMI 2 treated with 200 times the MIC of either ciprofloxacin (**A**) or penicillin (**B**). Bacteria were grown in liquid cultures and sampled at various times for luminescence (dashed lines) and viable counts (continuous lines). Antibiotics were added at time zero. Closed symbols represent untreated control cultures and open symbols antibiotic-treated cultures. **B** presents the mean ± SD of three independent experiments.

dent genetic loci, namely Tol103, Tol104, and Tol105 (Table 1).[2,3] Figure 4 indicates that, while these independent insertional mutants were virtually immune of penicillin-induced killing, they all demonstrated a disproportionate, sharp luminescence switch off during penicillin treatment. Thus, although luminescence correlated with both chloramphenicol-induced inhibition and ciprofloxacin-induced killing of *S. gordonii,* it did not correlate with penicillin-induced killing in this particular organism.

Studies on the discordance between penicillin-induced killing and luminescence

As already mentioned, bacterial chaining remained stable and thus could not account for such discrepant results. Because treatment with penicillin resulted in a rapid light switch off, we tested whether inactivating the drug *in situ* could reverse the phenomenon and switch the light on. Penicillin-treated cultures were supplemented or not supplemented with penicillinase (final concentration of 2000 U/ml; Bacto-Penase concentrate, Difco) 2 hr after the addition of antibiotics. Figure 5 indicates that while luminescence was rapidly switched off by penicillin, it was readily restored after penicillin inactivation with penicillinase. This ≥10 times luminescence "off/on" phenomenon was very discordant with the relatively low variation in viable counts during the same period of time. This suggested that penicillin had altered either luciferase, or some intracellular factor essential for luminescence, independently of bacterial viability.

This observation could be due either to luciferase degradation or to some metabolic alteration affecting the function of the enzyme. Both possibilities were tested in cell lysates. As in

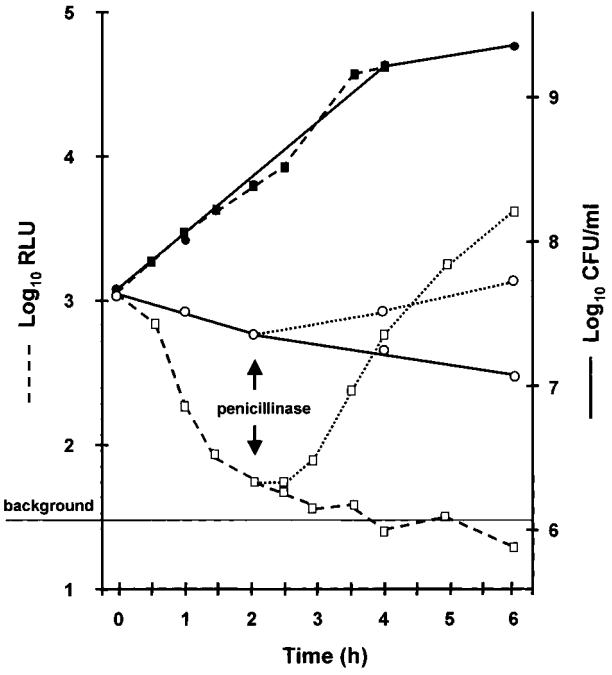


FIG. 5. Restoration of luminescence after inactivation of penicillin with penicillinase. Broth cultures of mutant LMI 2 were treated (open symbols) or not treated (closed symbols) with 200 times the MIC of penicillin, and both luminescence (dashed lines) and viable counts (continuous lines) were followed. Two hours after penicillin addition, the drug was inactivated with an excess of penicillinase (arrows). The prompt restoration of luminescence and the slow resumption of bacterial growth is depicted by the dotted lines.

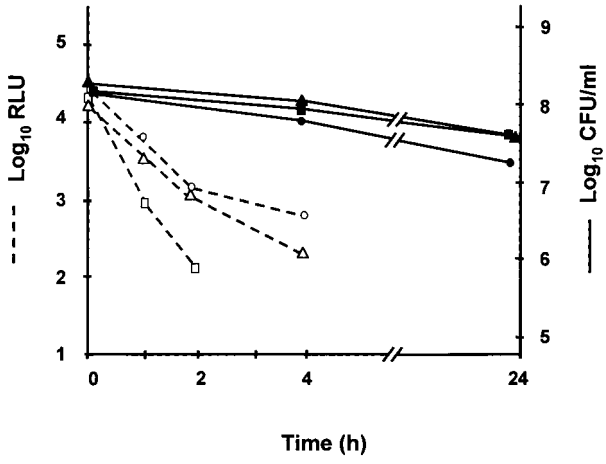


FIG. 4. Viable counts (closed symbols and plain lines) and luminescence (open symbols and dotted lines) of three tolerant mutants of *S. gordonii* carrying a luciferase reporter insert in various genes treated with 200 times the MIC of penicillin. Mutant Tol103 (squares) was described elsewhere,[2] and carries a luciferase-reporter insert in the *arcB* gene. Mutant Tol104 (triangles) has the same tolerant background as Tol103,[2] but carries the luciferase reporter insert of LMI2, transferred by DNA transformation. Mutant Tol105 (circles) has the same background as Tol103,[2] but carries a luciferase insert in the *scaA* gene.[3] Bacteria were processed as in Fig. 3. Penicillin was added at time zero.

the chloramphenicol experiment, Western blots indicated that luciferase remained present in the cells in spite of penicillin treatment (Fig. 6). Thus, the decrease of luminescence was not due to protein hydrolysis. On the other hand, luciferase could not be reactivated in lysates of penicillin-treated bacteria, in spite of ATP and pH adjustment. Thus, in contrast to the chloramphenicol experiment, luciferase was functionally inactive after penicillin treatment.

Penicillin-induced alteration of luminescence depends on de novo *protein synthesis*

The fact that the luminescence deficit persisted in cell extracts suggested the possible presence of an inhibitor. One candidate was penicillin itself. However, neither penicillin (2 mg/L), nor chloramphenicol (640 mg/L) affected the function of recombinant luciferase when added to the system *in vitro.* Another possibility was that bacteria treated with penicillin produced an inhibitor that was present in the cell lysates. A cross-inhibition of luminescence was sought by mixing cell extracts (final protein concentrations ranging from 2 to 80 μg/ml) from *S. gordonii* cultures exposed for 2 and 4 hr to penicillin with recombinant luciferase. No cross-inhibition was observed (data not shown).

An additional possibility was that penicillin induced some irreversible functional denaturation of luciferase. The question

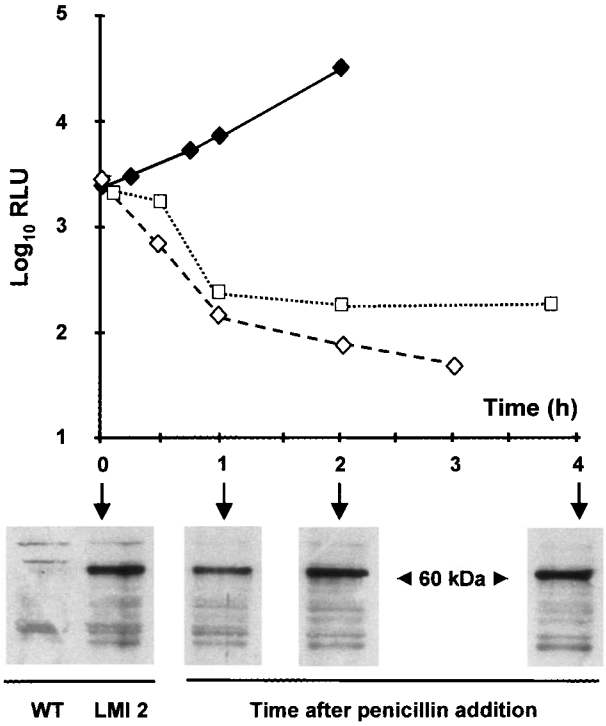


FIG. 6. The upper panel depicts the luminescence of whole cultures and cell extracts during penicillin treatment of mutant LMI 2. Bacterial cultures were treated (open symbols) or not treated (closed symbols) with 200 times the MIC of penicillin at time zero. Luminescence was determined in whole cultures (diamonds) or cell extracts (squares) at various times thereafter. The lower panel presents the amount of luciferase detected by Western blot in either the wild-type parent (WT) or cell extracts of mutant LIM 2 (Table 1) prepared 5 min before, or 1, 2, and 4 hr after drug addition. It can be seen that while luciferase was quantitatively stable, it was functionally unstable during penicillin treatment.

then arose as to whether the "off/on" phenomenon depended on *de novo* protein synthesis. This issue was addressed by adding chloramphenicol 5 min after penicillinase to penicillin-treated cultures. Figure 7 indicates that inhibiting *de novo* protein synthesis with chloramphenicol after penicillin inactivation blocked the restoration of luminescence. This supported the hypothesis that light restoration depended on newly made proteins.

DISCUSSION

The present experiments attempted to correlate the bacteriostatic and bactericidal effects of various drugs with luciferase activity in bioluminescent *S. gordonii*. The results indicated that the correlation depended on the drug class.

After addition of the protein inhibitor chloramphenicol, bacterial growth came to a halt. Light emission stopped increasing, and luminescence remained proportional to the culture viable counts. Moreover, luciferase could be recovered in a functional state from cell extracts. Thus, luciferase that had accumulated in the cells before antibiotic treatment remained both physically and functionally stable. Moreover, the surviving cells kept enough metabolic energy to activate luciferase. This was in agreement with previous studies reporting a "functional" half-life of luciferase varying between 2 and 4 hr after treatment of *S. mutans* and *M. smegmatis* with protein inhibitors including tetracycline, chloramphenicol, and streptomycin.[17,27,31]

When high concentrations of ciprofloxacin were used, the rapid decrease in bacterial viability was accompanied by a roughly parallel drop in luminescence. Because quinolones do not directly affect protein synthesis, the decrease in light emission was presumably due to killing-related de-energizing of the cells. This suggests that the system might be considered to follow the bactericidal effect of these kinds of molecules.

On the other hand, penicillin treatment resulted in a disproportional drop of luminescence when compared to viable counts. This contrasted with chloramphenicol and ciprofloxacin, and prohibited any correlation between light emission and the bactericidal effect of this particular drug. This discordance was observed both in the penicillin-killed LMI 2 and in a series of tolerant derivatives carrying *luc* reporter inserts in independent genetic loci. Thus, the phenomenon was related to penicillin treatment rather than to coincidental genetic constructs. Eventually, luminescence switch off did not result from alteration in luciferase transcription, because the protein was continuously present and physically stable after penicillin addition.

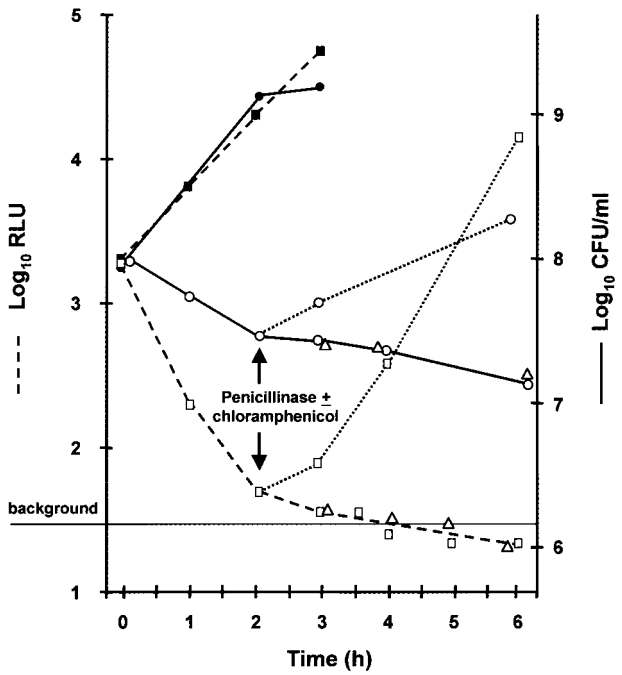


FIG. 7. The restoration of luminescence after penicillin inactivation is inhibited by chloramphenicol. The experiment of Fig. 5 was repeated. However, after penicillinase addition, the cultures were either left to recover as above (dotted lines) or supplemented with 20 times the MIC of chloramphenicol (open triangles). It can be seen that chloramphenicol blocked luminescence restoration.

Several factors might be incriminated in this luminescence defect. Previous studies showed that alterations in ATP or pH were critical.[6] In addition, products of the luciferase-luciferin reaction including ADP and inorganic phosphorus decrease luminescence as well.[6] However, ATP and pH were not responsible because adjustment of these parameters in cell extracts could not restore luminescence. Alternatively, penicillin treatment could have either induced the production of an inhibitor or promoted some kind of post-translational modification. Because the restoration of luminescence after penicillin inactivation required *de novo* protein synthesis, the hypothesis of a post-translational modification was the most likely one.

The penicillin-induced light switch off is reminiscent of the fact that inhibiting penicillin-binding proteins with β-lactams has a more complex physiological consequence than mere blockage of peptidoglycan assembly. The most dramatic irreversible consequence is the triggering of autolysins, resulting in wall degradation, cell lysis, and bacterial death.[33] However, this was not the case in the present study.

Reversible alterations include a number of metabolic changes that are independent of cell death. For instance, penicillin-treatment of *S. aureus, S. mutans,* and *S. pneumoniae* result in a rapid switch off of RNA and protein synthesis.[13,19,21] In *S. pneumoniae,* the decrease in macromolecule synthesis was identical in both wild-type and tolerant pneumococci, in spite of >3 log differences between viability losses after 4 hr of drug exposure.[19] Moreover, after inactivation of penicillin with penicillinase, protein synthesis was restored with a kinetic similar to that of luminescence in the luciferase-producing *S. gordonii.*[20] This suggests the existence of a "talk back" mechanism,[13] capable of blocking the synthetic apparatus in response to abnormal wall metabolism, irrespective of the bacterial survival.

Although this regulatory switch is not known, two β-lactam-dependent regulatory pathways were described recently. In enterobacteria, a complex of four determinants (AmpC, AmpR, AmpG, and AmpD) can sense the degradation of cell wall during β-lactam treatment, and derepress the chromosomal cephalosporinase AmpC.[14] In *S. aureus,* the transmembrane sensor-transducer (BlaRI) detects the presence of extracellular penicillin[35] and triggers the autocleavage of its cytoplasmic portion—a metalloprotease. The free metalloprotease then cleaves the penicillinase repressor (BlaI) and thus induced penicillinase (BlaZ).

In the present study, intracellular luciferase did not undergo proteolytic cleavage, as indicated by its unaltered electrophoretic mobility. However, other types of post-translational modifications might exist. Firefly luciferase belongs to a superfamily of adenylate-forming enzymes found in both eukaryotic and prokaryotic cells. It includes aminoacyl-tRNA synthetases, acyl-CoA ligases, gramicidine S and tyrocidine synthetases, and the first enzyme of the penicillin biosynthetic pathway.[32] Aminoacyl-tRNA synthetase and acyl-CoA ligase are involved in protein synthesis and energy metabolism. Such enzymes might be under the control of some transcriptional or translational regulation linked to macromolecule synthesis, and hence affected by penicillin. Because of its homology at the active site, the luciferase function might be down-regulated simultaneously with these enzymes.[4] The model is speculative and could include a number of metabolic and/or signaling intermediates. Some kind of post-translational modification would most likely fit with the observation described herein. Thus, introducing reporter enzymes sharing mechanistic homologies with normal metabolic reactions might reveal other effects than those expected.

In conclusion, while luminescence could be used as a global indicator for the bacteriostatic activity of antibiotics, it was not reliable to dissociate between growth inhibition and bacterial killing by penicillin. Recently, such a system was used to rapidly assess the bactericidal effect of a β-lactam in a model of experimental thigh infection in mice.[9] Decreased luminescence measured *in vivo* correlated with bacterial eradication. The present experiments suggest that luminescence can paradoxically decrease in inhibited, but nonkilled bacteria. Additional experiments with tolerant mutants would be useful to settle this issue. The killing-independent switch off of luminescence during penicillin treatment is intriguing. The observation stirs new thinking into pending questions regarding the "physiological" response of bacteria to cell wall inhibition.

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